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Exploring DNA methylation patterns in copper exposed *Folsomia candida* and *Enchytraeus crypticus*

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ABSTRACT

Accumulating evidence shows that epigenetics-mediated phenotypic plasticity plays a role in an organism's ability to deal with environmental stress. However, to date, the role of epigenetic modifications in response to stress is hardly investigated in soil invertebrates. The main objective of this proof of principle study was to explore whether total cytosine and locus-specific CpG methylation are present in two important ecotoxicological model organisms, the springtail *Folsomia candida* and the potworm *Enchytraeus crypticus*, and if so, whether methylation patterns might change with increased toxicant exposure. LC–MS/MS analyses and bisulfite sequencing were performed to identify the CpG methylation state of the organisms. We show here, for the first time, a total level of 1.4% 5-methyl cytosine methylation in the genome of *E. crypticus*, and an absence of both total cytosine and locus-specific CpG methylation in *F. candida*. In *E. crypticus*, methylation of CpG sites was observed in the coding sequence (CDS) of the housekeeping gene Elongation Factor 1α, while the CDS of the stress inducible Heat Shock Protein 70 gene almost lacked methylation. This confirms previous observations that DNA methylation differs between housekeeping and stress-inducible genes in invertebrates. DNA methylation patterns in *E. crypticus* were not affected by exposure to copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) mixed in with LUFA 2.2 soil at sublethal effect concentrations that decreased reproduction by 10%, 20% and 50%. Although, differences in CpG methylation patterns between specific loci suggest a functional role for DNA methylation in *E. crypticus*, genome-wide bisulfite sequencing is needed to verify whether environmental stress affects this epigenetic hallmark.

1. Introduction

Many studies have shown that natural populations contain a significant amount of genetic variation upon which environmental stress factors may act (Roelofs et al., 2008). For instance, a study on the springtail *Orchesella cincta* showed distinct stress-induced gene expression in animals not acclimated to metals. In contrast, significantly elevated constitutive as well as cadmium-induced metallothionein expression was found in animals that gained tolerance to metals, suggesting adaptation through increased constitutive cadmium detoxification (Roelofs et al., 2008). For a long time scientists have been focusing on genetic adaptation due to changes in nucleotide sequence of protein-encoding genes or their promoter regions. However, recent studies indicate that environmental stress can influence not only DNA sequences as such, but also the epigenetic markers associated with the DNA (Kille et al., 2013; Pierron et al., 2014). Epigenetics is the study of changes in the expression and function of genes that cannot be attributed to changes in DNA sequence (Richards et al., 2010; Head et al., 2012). Epigenetic markers that are associated with these changes include DNA methylation, histone modifications, nucleosome remodeling, and non-coding RNA-mediated transcriptional regulation (Fuks, 2005; Goldberg et al., 2007; Bossdorf et al., 2008). The most intensely investigated marker is DNA methylation, which consists of an addition of a methyl group (-CH3) to the fifth position at the cytosine nucleotide in DNA (Vandegehuchte and Janssen, 2011; Head, 2014).

DNA methylation is associated with a number of key processes such as development, genomic imprinting and cell differentiation (Regev et al., 1998; Marhold et al., 2004; Vandegehuchte et al., 2009a). A number of studies have provided convincing evidence that changes in DNA methylation patterns are associated with environmental stress and

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may result in physiological alterations. For example, exposure to soluble fractions of industrial waste resulted in significant DNA hypermethylation and induced oxidative damage in Nile tilapia (*Oreochromis niloticus*). Furthermore, increased rates of malondialdehyde (indicates the process of lipid peroxidation) in *O. niloticus* erythrocytes suggested a disturbance in lipid membranes in response to the contamination (Flohr et al., 2012). Another study showed that a contaminated environment contributed to a decrease in total cytosine methylation in the flatfish *Limanda limanda* and enabled the development of hepatocellular adenoma tumors (Mirbahai et al., 2011).

Adverse effects associated with altered methylation status have also been observed in invertebrates. For instance, genome-wide methylation analysis of *Daphnia magna* exposed to the toxic cyanobacterium *Microcystis aeruginosa* revealed a complex mechanistic response with differential methylation patterns enriched for serine/threonine amino acid codons and genes related to protein synthesis and transport (Asselman et al., 2017). Serine/threonine amino acids play an important role in stress response as they regulate protein folding. In response to *Microcystis* stress, differentially methylated genes corresponded with genes that are likely to be alternatively spliced. The authors suggested that DNA methylation plays an important role in the animal's response to toxicity as it is regulated by environmental stress (Asselman et al., 2017).

To date, the role of epigenetic modifications in response to stress is hardly investigated in soil dwelling organisms. Kille et al. (2013) reported epigenetic effects of environmental contamination (i.e. arsenic) on the earthworm *Lumbricus rubellus*. Methylation sensitive AFLP (Me-AFLP) analysis revealed a distinction between two lineages within the sampled earthworms. Lineage A did not show any association with soil arsenic concentrations. However, DNA methylation patterns were correlated with soil arsenic levels in lineage B, suggesting a function for DNA methylation in the adaptation to environmental contamination at least in some lineages.

In this proof of principle study, we aimed to explore whether total cytosine and locus-specific CpG methylation can be detected under normal homeostasis in the soil dwelling organisms *Folsomia candida* and *Enchytraeus crypticus*, and moreover, if methylation patterns might change with increased toxicant exposure. In more detail, we assessed whether CpG methylation patterns could be associated with the stress response to copper exposure, a metal that has previously been reported to cause toxic effects in springtails (Pedersen and Van Gestel, 2001) and enchytraeids (Gomes et al., 2012). Total cytosine methylation was measured as the ratio between deoxy guanosines and deoxy 5 methylated cytosines after total digestion of genomic DNA using liquid chromatography tandem mass spectrometry (LC–MS/MS). Additionally, CpG methylation of specific loci of gene bodies in the housekeeping gene elongation factor 1 alpha (Ef1α) and the stress-inducible gene heat-shock protein 70 (Hsp70) were investigated using bisulfite sequencing, as CpG methylation in invertebrates is mainly found in gene bodies (exons and introns), where housekeeping genes show in general elevated methylation levels associated with high constitutive expression (Lyko et al., 2010; Wang et al., 2013). Such data will indicate whether methylation could potentially vary between a housekeeping gene and a stress-response gene in our models. On the basis of these studies in invertebrates we hypothesize that housekeeping genes with high constitutive expression show high gene body methylation, while inducible stress response genes show lower levels of gene body methylation. Finally, we attempted to assess the influence of copper toxicity on the observed methylation patterns.

2. Materials and methods

2.1. Test compound and spiking of soil

Copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) (99% purity) was obtained from Merck (Darmstadt, Germany). LUFA 2.2 soil (Speyer, Germany) was used as a control/reference. This soil has a reported total organic carbon content of 2.09%, pH_{CaCl2} of 5.5 and a water holding capacity (WHC) of 46.5%. To obtain contaminated soils, LUFA 2.2 was spiked with dry powder using three effect concentrations (ECx) that affected reproduction by 10 (EC10), 20 (EC20) and 50 (EC50) percent compared to the untreated control. For *E. crypticus*, EC10, EC20 and EC50 values for the effect of $CuCl_2$ (literature data on $CuSO_4$ exposure in *E. crypticus* is absent) of 111, 159 and 293 mg Cu/kg dry soil were established from a range finding test (0–35–70–120–300 mg Cu/kg dry soil) performed by Menezes-Oliveira et al. (2011). Literature on $CuSO_4$ toxicity to *F. candida* showed an EC50 of 519 (CI: 21–13095) mg Cu/kg dry soil (Bruus Pedersen et al., 2000). To narrow down the range of the confidence intervals we performed a toxicity test with *F. candida* ourselves; see below. This test provided EC10, EC20 and EC50 values of 188, 319 and 782 mg Cu/kg, respectively. These values were used for further analyses. All soil samples were moistened with deionized water to reach a moisture content equivalent with 50% of the WHC.

2.2. Toxicity tests

The parthenogenetic springtail *F. candida* ("Denmark strain", VU Amsterdam) and the annelid worm *E. crypticus* (VU Amsterdam) were used as model organisms. The cultures of both species were kept in a climate room at 16 ± 0.5 °C and a 16:8 h light:dark regime.

A 28-day toxicity test with *F. candida* was performed following OECD guideline 232 (OECD, 2009), using 100 ml glass jars. Springtails were exposed to $CuSO_4{\cdot}5H_2O$ at concentrations of 0–100–200–400–800–1600 mg Cu/kg dry LUFA 2.2 soil in order to determine EC10, EC20 and EC50. For each treatment (control and all concentrations) five replicates were prepared. Springtail juveniles of synchronized age (10–12 days old) were used for the experiment. After two days of soil equilibration, ten animals were introduced into each test jar, which was closed with a plastic screw top. Once a week jars were aerated, soil was moistened with deionized water and animals were fed dried baker's yeast. Springtails were harvested by adding 100 ml of deionized water to each test jar, gently stirring and transferring them to a plastic beaker, allowing springtails to float on the surface. Pictures were taken of the surface, used later to count all animals with the software program ImageJ (version 1.49) so as to determine reproduction (number of juveniles). EC10, EC20 and EC50 values were determined by using a logistic dose response model; corresponding 95% confidence intervals were calculated by using non-linear regression analysis in IBM SPSS Statistics 23 software (Corp, 2015). Subsequently, a second toxicity test was performed similar to the previous test using the same number of replicates, where springtails were exposed to concentrations corresponding to these effect levels. After 28 days, juveniles produced from this second test were collected, snap frozen and stored at −80 °C for later epigenetic analyses.

The test with *E. crypticus* followed OECD guideline 220 (OECD, 2004). Animals were exposed to previously obtained effect concentrations (EC10, EC20 and EC50) (Menezes-Oliveira et al., 2011). Five replicates were prepared for each treatment (control, EC10, EC20 and EC50). Enchytraeids were selected based on their size (0.4–0.6 cm) and visible clitellum (indicating adulthood). Ten animals were introduced into each 100 ml glass jar containing 30 g of moist soil. Jars were closed with perforated aluminum foil and once a week all jars were aerated, soil moistened with deionized water and animals were fed with 2 mg oatmeal. After 3 weeks, tap water was added to jars containing enchytraeids, after which the animals could be collected from the water using a small hook. Collected enchytraeid juveniles were snap frozen and stored at −80 °C for later DNA analyses.

All toxicity tests were performed in climate room at 20 ± 0.5 °C, 75% relative humidity and a 16:8 h light:dark regime.

2.3. DNA isolation

Genomic DNA was extracted from about 100 frozen springtails or enchytraeid juveniles per biological replicate (1.5 ml microcentrifuge tube) using The Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). Animals were crushed with microfuge tube pestles in 100 µl of PBS buffer. To enable lysis, tissues were disrupted in 100 µl of Nuclei Lysis Solution and 2 µl of Proteinase K. Samples were incubated for 15 min at 60 °C in a water bath. After incubation, 170 µl of DNA lysis buffer was added to each tube and the samples were centrifuged at 14.000 rpm for10 min. After centrifugation, the supernatant was transferred to a DNA-binding mini-column and washed three times with 500 µl Wizard SV Wash Solution, by centrifuging each time at 14 000 rpm for 1 min. Finally, the DNA was eluted in 50 µl Nuclease-Free water and its concentration measured on a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA).

2.4. Total cytosine methylation (LC–MS/MS)

DNA samples were digested by diluting to 200 ng of genomic DNA in 10 µl TE buffer, adding, 10 µl of a mixture of benzonase (50 U/mL), phosphodiesterase (60 mU/mL) and alkaline phosphatase (40 U/mL) (Sigma, Germany) in buffer (20 mM TRIS, 100 mM NaCl and 20 mM $MgCl_2$, pH 7.9), with subsequent overnight digestion at 37 °C. After digestion, 80 µl of MilliQ water and 100 µl of internal standard solution (690 nM C10N5 dG, 41.4 nM D3 mdC, 1.38 nM D3 hmdC) were added to each sample. Finally, all digested samples were transferred to HPLC vials and injected in an Agilent 1200 µHPLC system, equipped with QQQ mass spectrometer (Agilent 6460). Single deoxynucleosides were separated with an Agilent ZORBAX Eclipse Plus C18 column (ID 2.1 x length 100 mm, particle size 1.8 µM). Mobile phases (0.1% formic acid in methanol and 0.1% formic acid in MilliQ water) were used in a linear gradient from 5% to 15% methanol within 3 min, with an additional equilibration at 5% methanol for a final 7 min. The following source conditions were applied during a LC–MS/MS run: gas temperature 350 °C, gas flow rate 10 l/min, nebulizer pressure 40 psi, capillary voltage + 4500 V. Scanning of deoxynucleosides occurred with a dwell time of 50 ms per compound using multiple reaction monitoring. The following mass transitions were used: 5mdC 242.1/126.1, D3mdC 245.1/129.1, dG 268.1/152.2, C10N5dG 243.3/162.2. The following fragmentation voltages were used: 76 V (5mdC and D3mdC), 65 V (dG and C10N5dG). A collision energy (voltage) of 8 V was used for 5mdC and D3mdC, whereas for dG and C10N5dG a collision energy of 4 V was used. A standard curve with increasing amounts of mC (0–2.07 µM, 0%-3%) was included against a fixed amount of dG (345 nM). Peak areas were acquired using manual integration in Agilent QQQ quantitative software. The amount of total cytosine methylation was determined by calculating the ratio to the corresponding internal standard, using the obtained peak areas ((Peak area mdC/peak area D3 mdC)/(Peak area dG/Peak area C10N5 dG)). These values were then interpolated with the calibration curve to actually derive a percentage of [mdC]/[dG].

2.5. Bisulfite sequencing

For gene-specific CpG methylation analysis we chose specific loci of gene bodies from a constitutively expressed housekeeping gene and an inducible stressor gene. The housekeeping gene of choice was the eukaryotic elongation factor 1 alpha (Ef1α), which binds aminoacyl tRNAs to the ribosome as part of the translation of mRNA. As an inducible stress-response gene we chose heat shock protein 70 (Hsp70), which plays a role in protein folding cell protection from stress. For each treatment (control, EC10, EC20 and EC50), three biological replicates were used for DNA isolation. For bisulfite conversion of genomic DNA (turning unmethylated cytosines into uracil and to thymine after PCR) from *F. candida* and *E. crypticus*, the Zymo EZ DNA Methylation-GoldTM kit (Ehrich et al., 2007) was used, following the manufacturer's protocol (Zymo Research, Orange, CA). Gene-specific primers were designed to target short fragments of Ef1α and Hsp70 for bisulfite treated (bs) genomic DNA and non-bisulfite treated (to verify the sequence) genomic DNA (Table S1). PCR reactions were set up as follows: 1 µl DNA template, 13.3 µl Nuclease-Free water (Promega), 5 µl 5 x PCR buffer, 1.5 µl $MgCl_2$, 2 µl dNTPs (2.5 mM of each dNTP), 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM) and 0.2 µl GOTaq polymerase (Promega) for a final volume of 25 µl. The following PCR program was used with annealing temperatures ranging from 54 to 61 °C: initial denaturation for 5 min at 95 °C, followed by 35 amplification cycles (30 s at 95 °C, 30 s at 54–61 °C and 1 min at 72 °C) and a final extension step of 1 min at 72 °C. For bisulfite-treated genomic DNA, the obtained fragments were validated for correct amplicon size with gel electrophoresis. Validated PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). DNA fragments were ligated into plasmid pGEM-T following the T4 DNA ligase protocol (Promega) and transformed into Escherichia coli XL-1 Blue following the Stratagene protocol (Stratagene). For each of the transformation reactions, positive transformation products were screened by PCR using the initial PCR primers to verify the insertion of the Ef1α and Hsp70 gene fragments. The fragments were visualized on a 1.5% (wt/vol) agarose gel. Positive transformants were cultured at 37 °C overnight, after which the plasmid DNA was isolated with the Wizard Plus SV Miniprep DNA Purification System (Promega). The purified plasmid DNA was sent to Eurofins for sequencing. For both *F. candida* and *E. crypticus* we obtained two sequences per biological replicate per gene. For non-bisulfite treated genomic DNA, amplification products were verified by electrophoresis on 1.5% (wt/vol) agarose gels and by staining with ethidium bromide. Validated samples were then used for direct sequencing (Eurofins).

2.6. Control DNA

To assure successful conversion of DNA with sodium bisulfite, we produced a negative control (completely demethylated DNA) and a positive control (completely methylated DNA). Demethylated genomic DNA was generated using the Qiagen Repli-g Mini Kit. The input was either 100 ng of *F. candida* or *E. crypticus* DNA. First, DNA was denaturated with buffer D1 during 3 min at room temperature. Buffer N1 subsequently stops denaturation. To generate purified DNA, a mix of nuclease free water, Repli-g mini reaction buffer and Repli-g DNA polymerase was added to the denatured DNA. This reaction was then incubated for 16 h at 30 °C and finally stopped by increasing the temperature to 65 °C to inactivate the enzymes. To remove potential contamination, the DNA was purified with phenol-chloroform. Finally, the purified DNA was eluted in 50 µl and measured on the Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA).

Completely methylated DNA was generated with a CpG methyltransferase (M.Sssl) and S-adenosyl methionine (SAM), allowing total methylation (100%) of DNA. M.Sssl (40U/µl) and SAM (32 mM) were used in a master-mix, which also contained 10 x NEB buffer 2, nuclease free water and DNA template. This master-mix was incubated for 2 h at 37 °C. After 2 h, M.Sssl and SAM were re-added, as these components got exhausted during incubation. Finally, the mixture was incubated for 4 h at 37 °C. DNA was purified with phenol-chloroform to remove possible contamination and finally eluted in 50 µl and measured on the Nanodrop.

2.7. Aligning sequences

CLC Genomic Workbench (Qiagen) was used to align non-bisulfite with bisulfite sequences for Ef1α and Hsp70 in both species. We used a very accurate (slow) progressive alignment algorithm designed by CLC, with the following parameters: Gap open-cost (10.0), Gap extension cost (1.0) and End gap cost (as any other place in the sequence).

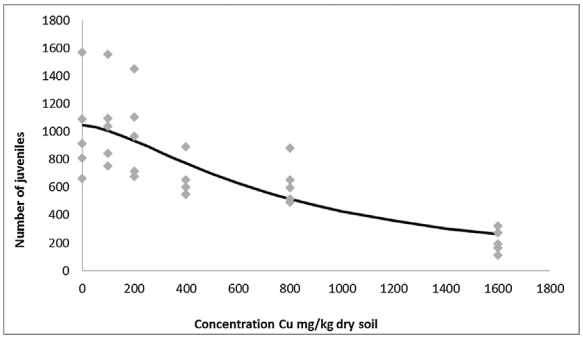

Fig. 1. Effect of $CuSO_4{\cdot}5H_2O$ on the reproduction (number of juveniles) of Folsomia candida after 28 days of exposure in LUFA 2.2 test soil. Line shows the fit of a logistic dose-response model to the data.

2.8. Statistical analysis

For *E. crypticus* a One-way analysis of variance (ANOVA) followed by the post hoc Dunnett's test was used to examine the significant effect of treatment (control, EC10, EC20 and EC50) on total cytosine methylation (Corp, 2015).

3. Results

3.1. Ecotoxicity of copper

For *F. candida* the average number of juveniles in the controls was 1006 per test jar (100% survival) with a coefficient of variance (CV) of 34.8%. Reproduction was reduced in a dose-related manner (Fig. 1) and EC10, EC20 and EC50 values (including 95% CI) obtained were 188 (0–426), 319 (28–610) and 782 (405–1159) mg Cu/kg dry soil, respectively. Effect concentrations for *E. crypticus* were previously established by Menezes-Oliveira et al. (2011) and EC10, EC20 and EC50 values including 95% CI were 111 (0.6–176), 159 (7.1–223) and 293 (200–937) mg Cu/kg, respectively.

3.2. Total cytosine methylation

LC–MS/MS chromatograms (Fig. 2) show the presence of methylated cytosines in the genome (5-mC residues) of *E. crypticus* with a small peak at 2.96 min retention time. The large peak at 4.76 min retention time represents deoxy-guanosine (all dGs in the genome). In contrast, *F. candida* is void of 5-mC residues. Further analysis revealed total cytosine methylation (expressed as%[mC]/[dG]) in *E. crypticus* with (mean ± SE) 1.41% ± 0.015 in control animals, 1.41% ± 0.014 in EC10 copper treatment, 1.38% ± 0.006 in EC20 and 1.40% ± 0.022 in EC50. Copper exposure did not significantly affect the rate of methylated cytosines in *E. crypticus* (ANOVA with Dunnett's method for multiple comparisons, $F = 0.95$; $p = 0.44$).

3.3. Bisulfite sequencing (PCR)

First, we verified the efficiency of bisulfite conversion using a positive and negative control sample provided by the manufacturer. Bisulfite conversion of genomic DNA was 100% in positive control samples, while a negative control showed a completely demethylated pattern (data not shown). Subsequently, bisulfite converted sequences (Ef1α and Hsp70) were aligned with non-bisulfite gene sequences obtained from direct sequencing. Fig. S1 represents a nucleotide alignment of bisulfite sequences with a reference non-bisulfite sequence of Ef1α locus in *E. crypticus*. Fig. S1 shows that in *E. crypticus* the sequenced Ef1α locus had 100% methylated CpG sites (9 sites per sequence) in 20 out of 24 sequences, whereas three sequences (k3.4, k12.5 and k12.8) showed 89% methylated CpGs and one sequence

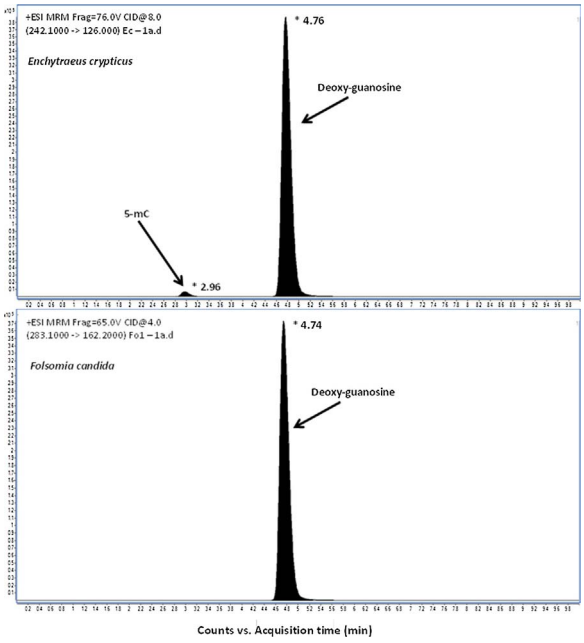

Fig. 2. LC–MS/MS chromatogram revealing 5-mC (total cytosine) methylation in *Enchytraeus crypticus* and an absence of methylation in *Folsomia candida*. The high peak at 4.76 (*E. crypticus*) and 4.74 (*F. candida*) minutes retention time accounts for deoxy-guanosine (all G's in the genome). The small peak at 2.96 min retention time accounts for 5-mC (methylated C's in the genome) in *E. crypticus*.

(k5.7) had 78% methylated CpG sites. These differences in methylation pattern were independent of copper treatment. The sequenced Hsp70 locus did not show methylation in 21 out of 24 sequences (Fig. S3). In 3 out of 24 cases some cytosines were still present after bisulfite sequencing but this is likely caused by an incomplete bisulfite conversion prior to sequencing. Again, all methylation patterns were independent of copper exposure. In the case of *F. candida*, both the Ef1α (Fig. S2) and the Hsp70 (Fig. S4) locus were void of CpG methylation. On the Ef1α locus, sequences k6.2 and k8.1 did show the presence of cytosines after bisulfite sequencing, as well as k1.2 on the Hsp70 locus, however, this is probably due to incomplete bisulfite conversion, as these cytosines were converted to uracil and were read as thymines in all other sequences.

In summary, for *F. candida* no peak of total cytosine methylation could be detected in the LC–MS profiles of copper-exposed animals. Also, no CpG methylation changes were detected in the gene sequences of Ef1α and Hsp70, again indicating that the measured methylation patterns are independent of copper toxicity in this study.

4. Discussion

A substantial number of studies have revealed an important role for DNA methylation in genome evolution and shifts in gene expression due to environmental stress (Gavery and Roberts, 2010; Glastad et al., 2013; Marsh and Pasqualone, 2014), mostly in vertebrate animals (Taudt et al., 2016). Therefore, the main objective for this study was to explore whether total cytosine and locus-specific CpG methylation are present in two invertebrate ecotoxicological model organisms and if so, whether methylation patterns might change with increased toxicant exposure.

Results obtained in this study showed that $CuSO_4$ affected *F. candida* reproduction with an EC50 of 782 (405–1159) mg Cu/kg dry soil. Effect concentrations that reduced reproduction (EC50 of 293 (200–937) mg Cu/kg) in *E. crypticus* were previously obtained by Menezes-Oliveira et al. (2011) and we observed a highly comparable decrease in enchytraeid reproduction at these concentrations in our study as well (data not shown).

While our ecotoxicity data revealed a response to copper exposure (i.e. decreased reproduction with increasing toxicity) in *F. candida*, we did not find either total cytosine methylation or locus-specific CpG methylation. Thus, the effect of copper toxicity could not be related to methylation patterns in the current study.

Although the LC–MS/MS method is able to measure methylated cytosines at trace levels, we cannot rule out that extremely scarce levels of cytosine methylation may be present in *F. candida*. Genome-wide bisulfite sequencing could eliminate this uncertainty. Although present in a substantial number of invertebrate taxa, DNA methylation and conventional DNA methyltransferases (DNMT1 or DNMT3) are absent in some invertebrates. A well-known example is the nematode *Caenorhabditis elegans* (Lyko and Maleszka, 2011). Despite the lack of this epigenetic machinery, recent papers report alternative epigenetic mechanisms in *C. elegans*, and suggest a role for histone modification (Vandegehuchte and Janssen, 2014; Greer et al., 2015) and piwi-interacting RNAs (Batista et al., 2008).

Another invertebrate, long assumed to lack DNA methylation, is the fruit fly *Drosophila melanogaster*. However, an increasing number of papers suggest that *D. melanogaster* actually does have cytosine methylation during early developmental stages, although these levels are low and difficult to detect (Glastad et al., 2011; Wang et al., 2013). Although low levels of cytosine methylation are found, *D. melanogaster* lacks DNA methyltransferase 1 (DNMT1) and 3 (DNMT3) necessary for establishing and maintaining cytosine methylation (Wang et al., 2013; Luo et al., 2015). The indicated absence of total cytosine and locus-specific CpG methylation in *F. candida* found in this study may be explained by the fact that homologs for DNMT1 or DNMT3 were not identified in the genome sequence (Faddeeva-Vakhrusheva et al., 2017). The near lack of DNA methylation or its complete absence in some invertebrates shows that methylation is not always essential for eukaryotic gene regulation (e.g., transcriptional regulation during development) (Glastad et al., 2011; Schübeler, 2015). When DNA methylation is lost during evolutionary time, organisms could use other epigenetic mechanisms to regulate gene expression (Raddatz et al., 2013).

As with *F. candida*, toxic effects of copper were observed in *E. crypticus* but in this case we did observe total cytosine methylation (~1.4%) in the genome. Although this level was considerably lower than described in other annelid species (e.g., 8.4% in the polychaete worm *Nephtys ciliata* and 13% in the earthworm *Aporrectodea caliginosa*) (Regev et al., 1998), the level of total cytosine methylation found in *E. crypticus* is comparable to the methylation levels observed in most insects (e.g., *Apis mellifera* 0.7%, *Schistocerca gregaria* 1.3–1.9%) (Regev et al., 1998; Lyko and Maleszka, 2011; Vandegehuchte and Janssen, 2014). We also found a high number of methylated cytosines in the gene body in a selected locus of the housekeeping gene Ef1α, whereas the inducible stress response gene Hsp70 revealed no gene body methylation. This is in line with other recent studies showing high gene body methylation in housekeeping genes with high constitutive expression and low levels or absence of gene body methylation in inducible stress response genes in other invertebrate species (Lyko et al., 2010; Wang et al., 2013). In our study, measured patterns of total cytosine and locus specific CpG methylation in *E. crypticus* were not correlated with copper toxicity, despite reported findings of decreased reproduction in enchytraeids as a consequence of increased copper concentrations (Gomes et al., 2012). Initially, we hypothesized that metals in general may affect methylation status, because this is supported by studies in vertebrates as well as invertebrates. Global DNA methylation patterns in *D. magna* were also not affected after exposure to increased concentrations of cadmium for two generations, despite reduction in reproductive output (Vandegehuchte et al., 2009b). However, in another study with zinc-exposed *D. magna*, a slight reduction in global DNA methylation was recorded (Vandegehuchte et al., 2009a). The fact that we did not observe such a relation between methylation and metal toxicity may be explained by the specificity of the stress response mechanisms induced by each metal. This was also suggested by transcriptome studies applying different metals at similar effect concentrations. For instance, Nota et al. (2010) used six different metals at EC10 and EC50 toxicity levels and found hardly any overlap in the gene regulatory networks that were regulated by the different metals. Only the well-known metal detoxification protein metallothionein showed consistent transcriptional activation in response to all tested metals. Therefore, copper may elicit molecular events that are very different from cadmium or zinc, and therefore may not affect methylation patterns. Environmental stress-induced DNA hypo-methylation patterns were also observed previously in the red flour beetle (*Tribolium castaneum*). Strong DNA demethylation of centromere sequences was associated with heat stress in this animal, indicating a role for cytosine methylation in heterochromatin structure remodeling during heat stress response (Feliciello et al., 2013). This could subsequently result in changes of chromatin-boundary activity and transcriptional activity of this region (Wong et al., 2006). To date, stress-induced hyper-methylation has not been observed in invertebrates. However, a number of studies have reported hyper-methylation in fish and plants. For example, increased global DNA methylation was observed in goldfish (*Carassius auratus*) exposed to cadmium, copper and zinc and was hypothesized to be involved in the organism's defence mechanism against metal stress (Vandegehuchte and Janssen, 2011).

5. Conclusion

With this exploratory study we suggest that *Folsomia candida* lacks total cytosine and locus-specific CpG methylation. We suggest that further investigation is necessary into alternative epigenetic mechanisms involved in gene regulation such as histone modifications. Total cytosine methylation (1.4%) and locus-specific CpG methylation were observed in *Enchytraeus crypticus*, but these methylation patterns did not change due to copper toxicity. Further research (i.e. genome-wide bisulfite sequencing) is necessary to obtain a global picture of DNA methylation in this model organism. This could also reveal the potential involvement of DNA methylation in the environmental stress response in these species. Finally, this study shows that DNA methylation differs not only between species but also between loci within a species, suggesting that epigenetic responses to environmental stress are not straightforward and cannot be generalised.

Competing interest

The authors declare no competing financial interest.

Supplementary information

Information about primer sequences and parameters (Table S1), and complete bisulfite sequencing alignments for the Ef1α (Fig. S1) and Hsp70 locus in E. crypticus (Fig. S3) and the same in F. candida (Figs. S2 and S4).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pedobi.2017.10.001.

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